REMARKS/ARGUMENTS

The Pending Claims

Claims 21, 60, and 70-89 are pending, which claims are directed to a polypeptide that comprises a particular amino acid sequence and binds with HIV gp120 under physiological conditions (claims 21, 70-77, and 86-89), and a composition comprising the polypeptide and a carrier (claims 60 and 78-85).

Amendments to the Claims

New claims 86-89 have been added, which are directed to a polypeptide comprising SEQ ID NOs: 12-15 with up to one conservative or neutral amino acid substitution. The new claims are supported by the claims as originally filed and by the specification, for example, at page 11, lines 16-18.

Summary of the Office Action

The Office rejects claims 21, 60, and 70-85 under 35 U.S.C. § 112, first paragraph, as allegedly lacking written description support. All other rejections have been withdrawn. Applicant requests reconsideration of this rejection.

Discussion of the Written Description Rejection

The Office rejects claims 21, 60, and 70-85 as lacking written description support for a polypeptide that *comprises* one of the four recited amino acid sequences, or an amino acid sequence of SEQ ID NOs: 1-12 *with up to 6 conservative or neutral amino acid substitutions*. The Office Action states three grounds for the rejection: (a) it is allegedly "well-documented" that single amino acid substitutions, even conservative in nature, can abrogate peptide activity, (b) Applicant has not provided evidence demonstrating that a reasonable number of polypeptide variants were prepared from SEQ ID NOs: 1-12 and assessed for biological activity, and (c) the previously submitted inventor's declaration failed to address the effects that flanking sequences might have on peptide activity.

As discussed in detail below, the Office has not cited any evidence or authoritative support for its position, and has not, therefore, met its burden of establishing a *prima facie* basis for rejection under Section 112. In the absence of a *prima facie* basis for the rejection, Applicant need not proffer any evidence to rebut the Office's allegations; the rejection falls on its own. Nevertheless, Applicant submits herewith a second inventor's declaration to further support the claims. Applicant's arguments and evidence submitted herewith and already of record are sufficient to rebut the Office's stated reasons for the rejection.

1. The Office Has Not Established a Prima Facie Case Under Section 112

There is a strong presumption that an adequate written description of the claimed invention is present when the application is filed. *In re Wertheim,* 541 F.2d 257, 263, 191 U.S.P.Q. 90, 97 (C.C.P.A. 1976); *In re Marzocchi,* 439 F.2d 220, 224, 169 U.S.P.Q. 367, 370 (C.C.P.A. 1971) (emphasis added). Furthermore, "it is incumbent upon the Patent Office ... to explain why it doubts the truth or accuracy of any statement in a supporting disclosure *and to back up assertions of its own with acceptable evidence or reasoning* which is inconsistent with the contested statement." *In re Marzocchi,* 439 F.2d at 224, 169 U.S.P.Q. at 370 (emphasis added). A general allegation of "unpredictability in the art" is not a sufficient reason to support a rejection for lack of adequate written description. *Id. See also, MPEP§ 2163.04*

The Office has not provided any evidence to support its basis for rejection. The Office alleges that it is "well-documented" that even a single conservative amino acid substitution can abrogate peptide activity; however, the Office does not cite a single authority for this proposition. The Office argues that Applicant has not provided examples of variants that maintain biological activity, but provides no supported reason to doubt the statements in the Application that such variants can be created. The Office alleges that Applicant's declaration fails to address the effects that flanking sequences might have on peptide activity, yet provides no reasoning that would suggest such flanking sequences are likely to disrupt peptide function.

The Office relies solely upon unsupported allegations as a basis for its rejection, improperly attempting to shift the burden of proof to the Applicant. Thus, the Office has failed to set forth a *prima facie* case in support of the Section 112 rejection.

2. The Claims Satisfy the Written Description Requirement of Section 112

Although the burden of proof has not shifted to Applicant to overcome the ill-supported rejection, Applicant has nevertheless provided sufficient reasons and evidence to do so in this and earlier communications to the Office. In this regard, Applicant reasserts its prior arguments as to the Section 112 rejection, and provides the following additional comments.

Applicant previously submitted evidence, by way of the Declaration of Carl Saxinger, Ph.D. dated July 31, 2005, that one of ordinary skill in the art reading the application is armed with the knowledge of the physical and chemical properties of the amino acid residues that are available, and can readily discern which amino acids have similar chemical properties such that they would be suitable for substitution (see Declaration of Carl Saxinger, Ph.D. dated July 31, 2005, at paragraph 4; specification at page 7, line 12, through page. 8, line 11). The prior declaration also stated that an amino acid sequence containing one or more conservative substitutions retains chemical and physical properties similar to the amino acid sequence upon which it is based, and that a conservatively substituted sequence is expected to retain the function of the sequence upon which it is based at least to some degree (see Declaration of Carl Saxinger, Ph.D. dated July 31, 2005, at paragraphs 5-6).

The prior and present declarations explain that the Examples provided in the application demonstrate not only that the claimed sequences bind gp120, but that particular portions of the claimed sequences bind gp120 to a greater or lesser degree, or not at all (see Declaration of Carl Saxinger, Ph.D. dated July 31, 2005, at paragraphs 7-8; Declaration of Carl Saxinger, Ph.D. dated May 23, 2006, at paragraph 3). This information, as read by one of ordinary skill in the art, provides specific guidance as to which amino acid residues of the claimed sequences are candidates for substitution without abrogating binding activity (see Declaration of Carl Saxinger, Ph.D. dated May 23, 2006, at paragraph 4).

The application, thus, adequately describes a variant of SEQ ID NOs: 12-15 with up to six conservative or neutral amino acid substitutions, and certainly provides an adequate description of such variants comprising up to one conservative or neutral amino acid substitution as recited in the new claims.

Regarding the effect of flanking sequences on the binding activity of SEQ ID NOs: 12-15, the incorporation of biologically active sequences into larger molecules was state of the art at the time the application was filed (see Declaration of Carl Saxinger, Ph.D. dated May 23, 2006, at paragraphs 9-10). It would be the rare instance that a flanking sequence would be chosen that would have the necessary primary, secondary, and tertiary structure needed to abrogate peptide function (see Declaration of Carl Saxinger, Ph.D. dated May 23, 2006, at paragraph 11). This is especially true given the general knowledge and tools available at the relevant time (see Declaration of Carl Saxinger, Ph.D. dated May 23, 2006, at paragraph 11). Furthermore, it was within the skill of the ordinary researcher during at the relevant time to chose effective flanking sequences and test such constructs using no more than routine experimentation (see Declaration of Carl Saxinger, Ph.D. dated May 23, 2006, at paragraph 12).

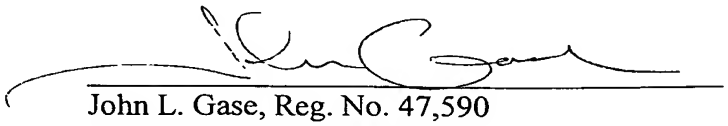
Moreover, contrary to the Office's assertions, the Examples provide evidence of the effect that flanking sequences would have on SEQ ID NOs: 12-15 in at least two respects. First, the sequences described in the Examples were tested by linking the sequences to a larger polylysine backbone molecule (see Declaration of Carl Saxinger, Ph.D. dated May 23, 2006, at paragraph 5). Thus, the tested sequences, in fact, contained one or more "flanking" amino acid residues (see Declaration of Carl Saxinger, Ph.D. dated May 23, 2006, at paragraph 5). Furthermore, the sequences were tested as a transitioning sequence offset format. In other words, each sequence tested contained part of the preceding sequence to which four additional amino acids are added to the carboxyl-end of the sequence, and four amino acids are effectively removed from the amino-end of the sequence (see Declaration of Carl Saxinger, Ph.D. dated May 23, 2006, at paragraphs 6-7). In this respect, the Examples demonstrate the effect of flanking sequences (see Declaration of Carl Saxinger, Ph.D. dated May 23, 2006, at paragraphs 6-7).

For the foregoing reasons, the subject matter of the pending claims meets the written description requirement of Section 112, first paragraph. Accordingly, the rejection should be withdrawn.

Conclusion

Applicants respectfully submit that the patent application is in condition for allowance. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,



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Date: May 23, 2006

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Saxinger

Art Unit: 1648

Application No. 10/084,813

Examiner: Jeffrey S. Parkin

Filed: February 27, 2002

For: POLYPEPTIDES THAT BIND HIV gp120 AND RELATED NUCLEIC ACIDS, ANTIBODIES, COMPOSITIONS, AND METHODS OF USE

DECLARATION UNDER 37 C.F.R. § 1.132 OF CARL SAXINGER, PH.D.

I, Carl Saxinger, Ph.D. do hereby declare that:

1. I am the sole inventor of the subject matter disclosed and claimed in the instant application. I have been actively engaged in research in the relevant art since a time prior to the filing of the instant application, and I am familiar with level of skill in the art and the general knowledge that was available to the person of ordinary skill in the art at that time.

2. The pending claims of the instant application are directed to a polypeptide that comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 12-15, with up to 6 conservative or neutral amino acid substitutions. The claims require that the polypeptide binds with HIV gp120 under physiological conditions.

3. As described in my prior Rule 132 Declaration dated July 31, 2005, the instant application provides guidance as to which amino acids are required for binding activity. In particular, Example 1 illustrates that SEQ ID NOs: 12-15 bind with high affinity to HIV gp120. Example 1 also shows that amino acid sequences comprising only certain portions of SEQ ID NOS: 12-15 also bind to HIV gp120 with relatively high affinity, but that other amino acid sequences that comprise a different portion of SEQ ID NOs: 12-15 do not share the same binding affinity with HIV gp120. The sequences tested in Example 1 correspond to SEQ ID NOs: 12-15 as follows: SEQ

ID NOs: 50-59 correspond (in part) to SEQ ID NO: 12; SEQ ID NOs: 73-81 correspond (in part) to SEQ ID NO: 13; SEQ ID NOs: 89-98 correspond (in part) to SEQ ID NO: 14; SEQ ID NOs: 101-109 correspond (in part) to SEQ ID NO: 15. A more detailed correlation of the tested sequences to the claimed sequences is provided in the prior Rule 132 Declaration.

4. By comparing the tested sequences that retain binding affinity to those that do not retain binding affinity, one of ordinary skill in the art during the relevant time reading the application would have been able to discern which amino acid residues of SEQ ID NOS: 12-15 are required for binding activity. For example, from the information on binding activity provided in Example 1, as summarized above, the ordinarily skilled artisan would have been able to determine that the residues "LLTG" of SEQ ID NO: 12, the residues "SQYQ" of SEQ ID NO: 13, and the residues "LLNT" of SEQ ID NO: 14 are most important for binding activity. Similarly, the data shows that SEQ ID NO: 15 contains two sub-sequences of residues involved in binding "FVGE" and "FFQK" contained within the larger sub-sequence "FVGEKFRNYLLVFFQK." Example 1, thus, provides specific guidance as to which amino acid residues are the most likely candidates for conservative or neutral amino acid substitutions.

5. Example 1 of the instant application also provides some evidence of binding activity in the presence of flanking sequences in at least two respects. First, the binding activity of the receptor peptides described therein was conducted in the context of a 100 unit long polylysine backbone. The procedure used is similar to that discussed in Saxinger et al., *BMC Immunology, 6,* 1-15 (2005) (copy enclosed). In this respect, every sequence described in Example 1 was tested as part of a larger construct with a "flanking" lysine connecting the sequence to a polylysine molecule.

6. Second, the sequences described in Example 1 were tested as a transitioning sequence offset (four amino acids offset at a time) to show which sequence motifs were most important to the binding activity. This is apparent by reading down the list of sequences tested in Example 1, wherein each successive sequence contains part of the sequence before it modified such that four amino acids are removed from the amino-end of the sequence, and four amino acids are added to the carboxy-end of the sequence.

7. In this respect, Example 1 demonstrates the addition of "flanking" amino acids to the binding sequences of the molecule by effectively adding amino acids to one end of the claimed sequences, while removing amino acids from the other. For instance, SEQ ID NO: 53 tested in Example 1 shows that adding the amino acid residues "YAAA" to the amino-end of SEQ ID NO: 12 does not abrogate binding activity. Similarly, SEQ ID NO: 56 tested in Example 1 shows that adding "GIFF" to the carboxy-end of SEQ ID NO: 12 does not abrogate binding activity. As the transitioning of SEQ ID NO: 12 progresses (e.g., to SEQ ID NOs: 52 or 57, the amino acids important for binding (e.g., "SYQY") are removed, thereby abrogating activity. Similarly, SEQ ID NOs: 92 and 95 tested in Example 1 show the effect of adding, respectively, the sequences "FLFW" to the amino-end of the SEQ ID NO: 14 and "SSNR" to the carboxyl-end of the SEQ ID NO: 14. SEQ ID NOs: 74-76 tested in Example 1 show the effect of adding amino acids to the amino-end of SEQ ID NO: 13, and SEQ ID NOs: 103 and 104 show the effect of adding amino acids to the amino-end of SEQ ID NO: 15. No examples, however, are provided that show the effect of adding amino acids to the carboxyl terminus of SEQ ID NOs: 13 or 15 because, as transitioning progressed, the amino acids most involved in binding that were located near the amino terminus were removed, thereby abrogating binding activity.

8. Furthermore, it was well within the skill of the ordinary researcher at the relevant time to determine whether any given flanking sequence would disrupt the biological activity of SEQ ID NOs: 12-15 using no more than routine experimentation and the general knowledge then available.

9. During the relevant period of time, research scientists routinely placed core binding sequences, such as SEQ ID NOs: 12-15, within a larger molecule for a variety of reasons. For example, larger polypeptides could be designed to include multiple copies of a sequence of interest so as to create a molecule with increased binding activity. Also, facilitating or associating sequences generally known in the art at the time could be placed in proximity to the core sequence to modulate the activity of the core sequence. Similarly, known targeting sequences could be placed in proximity to the core sequence to direct the polypeptide to a particular tissue or cell type. Furthermore, increasing the length of the polypeptide can stabilize the core sequence (e.g., by increasing *in vivo* half-life).

10. Examples of such constructs, whereby a core sequence with an identified biological activity is utilized as part of a larger construct, are commonplace in the art (e.g., Reeves et al., *J. General Virology, 79,* 1793-99 (1998) (copy enclosed)).

11. It would be the rare instance, that a chosen flanking sequence would have exactly the primary, secondary, and tertiary structure needed to block the activity of the core binding sequence. Furthermore, during the relevant period of time, the effect of a given flanking sequence on the binding activity of the core sequence could be predicted based on the chemical properties of the amino acid residues involved. These chemical properties are widely known and well understood, and have been since a time long before the present application was filed. Indeed, computer modeling programs were readily available during that time that could show any predictable conformational changes that would be caused by the addition of flanking sequences.

12. Thus, it was well within the skill of the ordinary researcher at the relevant time to choose appropriate flanking sequences that would not abrogate the binding activity of SEQ ID NOs: 12-15. After selecting appropriate candidate flanking sequences, routine and simple tests could have been used by such a researcher to confirm that the molecule retains the intended biological properties.

13. I hereby each declare that all statements made herein of our own knowledge are true, that all statements made on information and belief are believed to be true, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 5/23/06

Carl Saxinger

Carl Saxinger, Ph.D.

Journal of General Virology (1998), 79, 1793–1799. Printed in Great Britain

SHORT COMMUNICATION

The second extracellular loop of CXCR4 is involved in CD4-independent entry of human immunodeficiency virus type 2

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Human immunodeficiency virus type 2 (HIV-2) strains that infect cells in the absence of cellular CD4 emerge spontaneously *in vitro* after culture in CD4 T-cell lines. The HIV-$2_{ROD/B}$ strain can use the CXCR4 chemokine receptor for efficient entry into CD4 cells. Here we have shown that the rat homologue of CXCR4, in the absence of CD4, failed to mediate CD4-independent entry by ROD/B. Furthermore, using rat–human chimeric CXCR4 receptors we have demonstrated that the second extracellular loop (E2) of human CXCR4 is critical for HIV-2 infection of CD4 cells. E2 is also important for HIV-1 infection of CD4 cells. Our results therefore indicate that the role of E2 in HIV entry is conserved for HIV-1 and HIV-2 and for infection in the presence or absence of CD4.

Primate immunodeficiency viruses [human immunodeficiency virus types 1 and 2 (HIV-1, -2) and simian immunodeficiency virus (SIV)] enter cells by a process of membrane fusion triggered by the interaction of their envelope glycoproteins (Env) with the cellular receptor complex (reviewed in Moore *et al.*, 1993; Weiss, 1993). CD4 acts as a receptor for the outer envelope glycoprotein (SU) and is responsible for induction of conformational changes in Env. Several seven-transmembrane domain spanning molecules belonging to the chemokine receptor family have been identified as coreceptors (or second receptors) for HIV entry (reviewed in Clapham & Weiss, 1997; D'Souza & Harden, 1996; Moore *et al.*, 1997). Chemokine receptors CXCR4 and CCR5 are major coreceptors for T-cell-line-tropic and macrophage-tropic HIV-1 isolates respectively (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Doranz *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996). Other seven-transmembrane receptors can also mediate HIV-1 entry, namely CCR2b, CCR3, STRL33/Bonzo/TYMSTR, GPR15/BOB and the human-cytomegalovirus-encoded US28 (Choe *et al.*, 1996; Deng *et al.*, 1997; Doranz *et al.*, 1996; Farzan *et al.*, 1997; Liao *et al.*, 1997; Loetscher *et al.*, 1997; Pleskoff *et al.*, 1997*b*). Like HIV-1, HIV-2 requires a coreceptor for entry into $CD4^+$ cells (Clapham *et al.*, 1991; Dragic & Alizon, 1993; McKnight *et al.*, 1994). CXCR4 and CCR5 function as coreceptors for different strains of HIV-2 (Deng *et al.*, 1997; Hill *et al.*, 1997; McKnight *et al.*, 1998; Pleskoff *et al.*, 1997*a*, *b*; Sol *et al.*, 1997) as well as other members of the seven-transmembrane receptor family (Deng *et al.*, 1997; Farzan *et al.*, 1997; McKnight *et al.*, 1998). Feline immunodeficiency virus (FIV) is able to fuse human cells expressing CXCR4 or its feline homologue (Willett *et al.*, 1997*a*, *b*), suggesting an evolutionary link between chemokine receptor utilization and lentivirus infection.

Certain HIV-2 strains, adapted *in vitro*, infect $CD4^-$ cells (Clapham *et al.*, 1992; McKnight *et al.*, 1994) and use CXCR4 as a primary receptor (Endres *et al.*, 1996; Potempa *et al.*, 1997; Reeves *et al.*, 1997), presumably by direct interaction. In addition, some HIV-2 strains can be activated by soluble CD4 (sCD4) to use other chemokine receptors, namely CCR3 and V28 as well as CXCR4, for entry into $CD4^-$ cells, albeit less efficiently (Reeves *et al.*, 1997).

We previously reported that the N-terminal extracellular domain and the glycosylation state of CXCR4 influence infection and fusion of certain HIV-1 and HIV-2 strains (Brelot *et al.*, 1997; Picard *et al.*, 1997; Potempa *et al.*, 1997; Talbot *et al.*, 1995), and that the rat homologue of CXCR4 is not functional for CD4-dependent entry of some isolates of HIV-1 and HIV-2_{ROD} (Pleskoff *et al.*, 1997*a*). The second extracellular loop of human CXCR4 confers CD4-dependent infection for several HIV-1 strains when placed into a rat CXCR4 background (Brelot *et al.*, 1997). Here we show that the same region is also critical for CD4-independent infection by HIV-2.

HIV-$2_{ROD/B}$ can infect a number of $CD4^-$ human cell lines (Clapham *et al.*, 1992; McKnight *et al.*, 1994; Talbot *et al.*,

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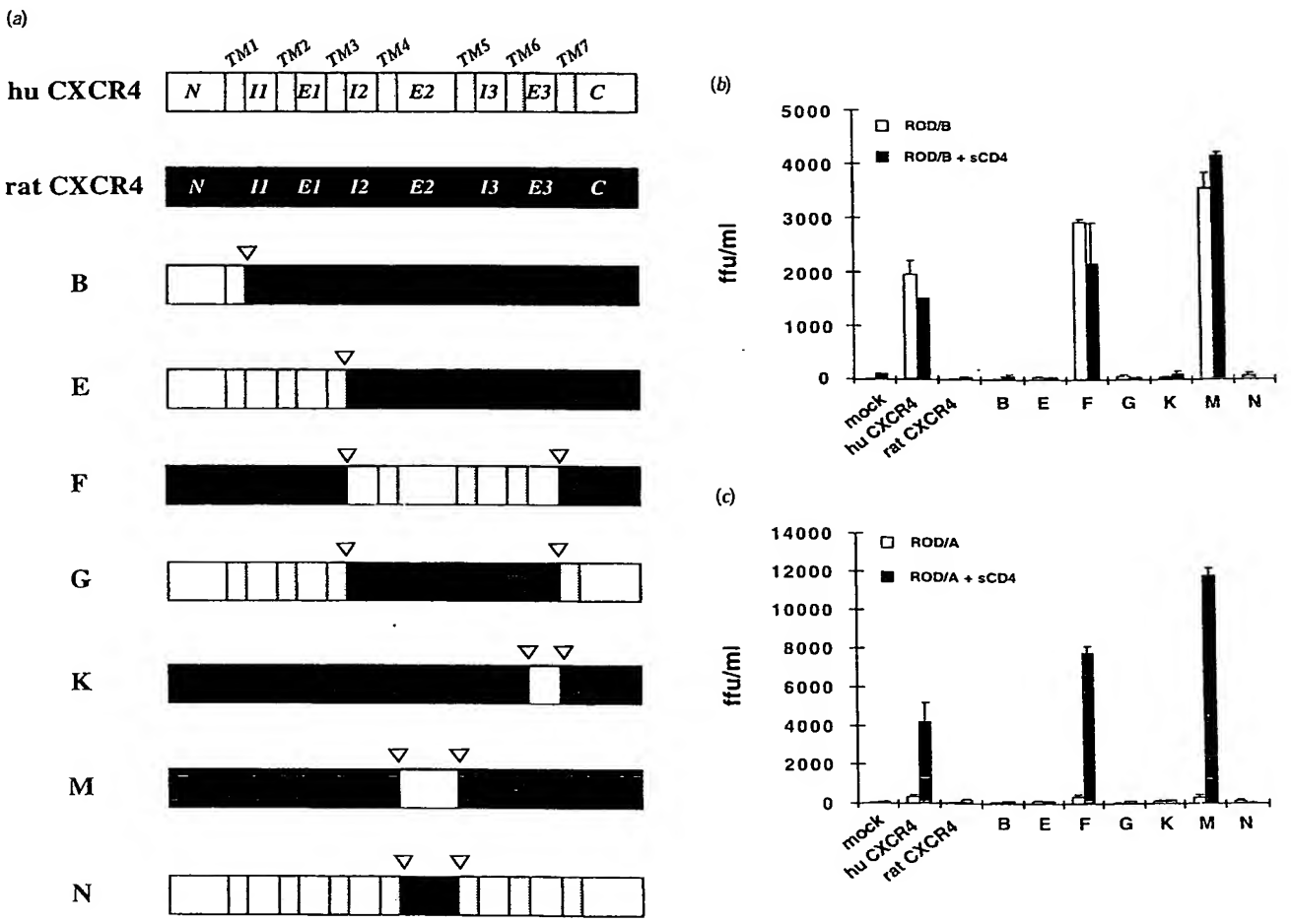

Fig. 1. (*a*) Schematic representation of human–rat CXCR4 chimeras. Segments of human origin are open while segments of rat origin are filled. Transmembrane domains are shaded and triangles indicate junction sites. Construction of these chimeric receptors has been previously described in detail (Brelot *et al.*, 1997). (*b*, *c*) Infectious titres of HIV-$2_{ROD/B}$ (*b*) and HIV-$2_{ROD/A}$ (*c*) on CCC cells transfected with human CXCR4, rat CXCR4 and chimeric receptors (B–N) in the presence or absence of sCD4 (5 μg/ml). Infectivity was quantified 3 days post-infection by immunostaining for expression of envelope glycoproteins. Results presented are from a representative experiment and are expressed as focus forming units/ml (f.f.u./ml) ± SD, determined on duplicate wells.

1995). Non-human cells are generally restrictive to CD4-independent entry of ROD/B, although some, e.g. the cat kidney CCC cell line used here, are permissive for post-entry replication (Clapham *et al.*, 1992; McKnight *et al.*, 1994).

CCC cells were seeded in 50 mm diameter dishes (10^6 cells per dish) and transfected 24 h later with 3 μg of pRcCMV plasmid encoding CXCR4 receptors (Fig. 1*a*; Brelot *et al.*, 1997) using Lipofectamine (Gibco BRL). Twenty-four hours post-transfection, cells were split into 48-well trays (5×10^4 cells per well) and infected with cell-free virus supernatant 24 h later. Infections were performed in duplicate with serially diluted virus in the presence or absence of 5 μg/ml sCD4. Virus (100 μl) was incubated with cells for 3 h at 37 °C before addition of 500 μl fresh medium. Cells were immunostained for envelope expression 3 days post-infection (Reeves *et al.*, 1997).

Expression of human CXCR4 in CCC cells confirmed sensitivity to HIV-$2_{ROD/B}$ infection (Fig. 1*b*), indicating that CXCR4 acts as a primary receptor in these cells. Addition of sCD4 did not influence CXCR4-mediated entry of HIV-$2_{ROD/B}$ but activated efficient infection of the prototype HIV-$2_{ROD/A}$ strain (Fig. 1*c*). Marginal infection by this strain in the absence of sCD4 was enhanced approximately 10-fold by 5 μg/ml sCD4, consistent with previous reports (Endres *et al.*, 1996; Potempa *et al.*, 1997; Reeves *et al.*, 1997). In contrast, the rat homologue of CXCR4 did not mediate entry of ROD/A or ROD/B (Fig. 1*b*, *c*). Since this molecule was expressed at

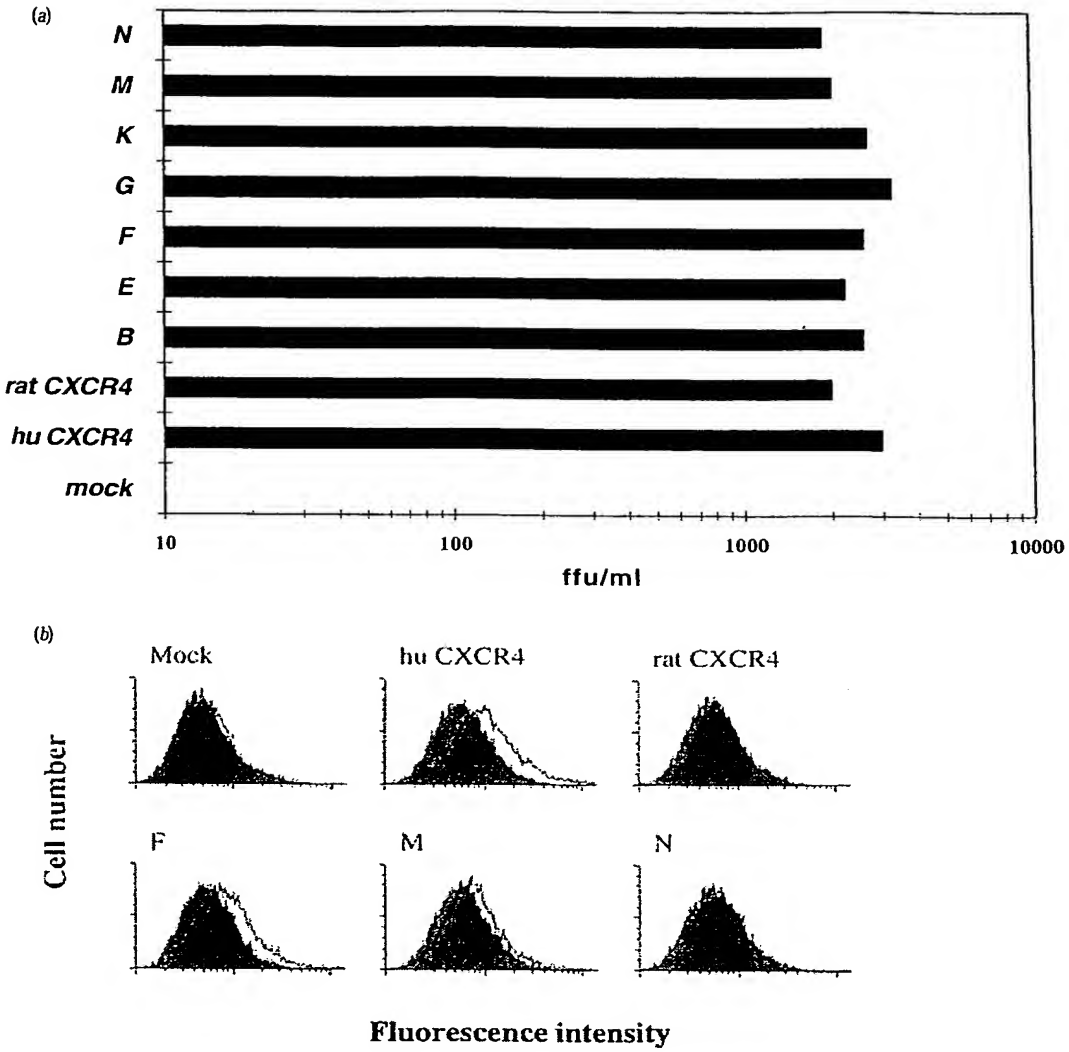

Fig. 2. (*a*) Infectious titres of HIV-1_{LAI} on CCC/CD4 transiently transfected with human CXCR4, rat CXCR4 and chimeric receptors (B–N). (*b*) FACS data showing 12G5 binding to mock, human CXCR4 (hu CXCR4), rat CXCR4, chimera F, M and N transfected $CD4^-$ CCC cells. The shaded area represents isotype binding and the unfilled area 12G5 binding.

similar levels to human CXCR4 on transfected cells (see below), we concluded that rat CXCR4 is not functional as a receptor for HIV-2_{ROD} in $CD4^-$ cells. Human and rat CXCR4 sequences (Wong *et al.*, 1996) are closely related (greater than 90% amino acid identity). Differences are mainly found in extracellular domains, in particular in the N-terminal extracellular domain (Nt) (11 aa differences) and in the second extracellular loop (E2) (8 aa differences), suggesting a role for these regions in HIV (co)receptor function (Brelot *et al.*, 1997).

We next analysed receptor chimeras between rat and human CXCR4 (Fig. 1*a*) for the ability to mediate HIV-2 infection following transfection into CCC cells (Fig. 1*b*, *c*). Introduction of the Nt and transmembrane (TM) region 1 (construct B) or a fragment encompassing the Nt and the extracellular loop 1 (E1) (construct E) of human CXCR4 into the non-functional rat homologue did not restore receptor function. Conversely, replacement of the Nt and TM1 fragment of human CXCR4 by its rat counterpart did not abrogate entry (not shown). Thus, although the Nt is essential for ROD/B cell–cell fusion and modulates cell-free infection of HIV-$2_{ROD/B}$ to a lesser extent (Potempa *et al.*, 1997), sequence differences between rat and human E2 were not detrimental to the receptor function of CXCR4. We next exchanged a fragment that included both E2 and E3 extracellular loops between rat and human CXCR4. Introduction of this fragment of human CXCR4 into rat CXCR4 (construct F) created a

functional receptor for HIV-$2_{ROD/B}$ entry and for HIV-$2_{ROD/A}$ entry following sCD4 activation (Fig. 1*b*, *c*). The reciprocal chimera (construct G) did not function as a receptor. These results indicated that the region(s) important for CXCR4 receptor activity are within the I2-to-E3 fragment. The two human extracellular domains of this fragment (E2 and E3) were therefore introduced individually into rat CXCR4 (constructs M and K respectively). E3 alone did not restore receptor activity to rat CXCR4 (construct K). Conversely, replacing human E3 with rat E3 did not affect receptor activity of human CXCR4 (not shown). In contrast, replacement of rat E2 by human E2 created a functional receptor for HIV-2 (construct M), whereas the reciprocal construct (N) was unable to mediate HIV-2 entry into CCC cells. The second extracellular loop of CXCR4 is thus a crucial determinant for its ability to mediate HIV-2 entry in the absence of cellular CD4. Interestingly, infection via chimeras F and M was approximately 2- and 3-fold higher, respectively, than infection mediated by human CXCR4, perhaps suggesting differences in surface expression of parental and chimeric receptors. However, this seemed not to be the case when we went on to examine cell-surface expression of CXCR4 receptors (see below).

Since monoclonal antibodies that recognize both rat and human CXCR4 have not yet been identified, we assessed cell-surface expression of parental and chimeric CXCR4 receptors in two ways. First, we tested whether chimeric receptors, expressed after transfection, supported HIV-1_{LAI} infection on CCC/CD4 cells. HIV-1_{LAI} is able to use human CXCR4, rat CXCR4 and rat–human chimeras for efficient entry into $CD4^+$ cells (Brelot *et al.*, 1997). All the chimeric receptors supported HIV-1_{LAI} infection (Fig. 2*a*) when transiently transfected into CCC/CD4 cells. The levels of infection were similar between the different receptors, indicating that sufficient amounts of each receptor were expressed on $CD4^+$ CCC cells to allow LAI infection. Infections and staining were performed as described for CCC cells with the exception that p24 antigen expression (instead of Env) was determined (Simmons *et al.*, 1996). Secondly, we assessed cell-surface expression of the rat–human chimeras on CCC cells using monoclonal antibody 12G5, which binds to human but not rat CXCR4, and recognizes a conformational epitope, including the E2 domain (Brelot *et al.*, 1997; Endres *et al.*, 1996; Lu *et al.*, 1997). Cell-surface expression of CXCR4 constructs was determined by fluorescence-activated cell sorting (FACS), as previously described (Potempa *et al.*, 1997), 2 days post-transfection. FACS analysis confirmed that the epitope of 12G5 includes E2 of human CXCR4, with receptor constructs F and M recognized by the 12G5, albeit less efficiently (Fig. 2*b*). Since constructs F and M are at least as effective as human CXCR4 as a primary receptor for HIV-2 (Fig. 1*b*, *c*) and as a coreceptor for CD4-dependent HIV-1_{LAI} (Fig. 2*a*), the reduced 12G5 signal may reflect a lower 12G5 affinity to F and M and not a reduced surface expression. Receptors containing rat E2 were not recognized by 12G5 (Fig. 2*b* and data not shown).

We went on to examine inhibition of ROD infection by 12G5 and SDF-1α. The anti-CXCR4 12G5 MAb is able to neutralize CD4-independent Env-induced cell fusion of HIV-2 (Endres *et al.*, 1996; McKnight *et al.*, 1997). Fig. 3 (*a*) shows that HIV-2_{ROD} infection, mediated by CXCR4 in the absence of cellular CD4, was inhibited in a dose-dependent manner by 12G5. HIV-2 ROD/A and ROD/B infection through human CXCR4 was inhibited approximately 65–85% at 10 μg/ml of 12G5. For infection via construct M, however, 12G5 inhibition was much weaker, with only approximately 23–33% of infection inhibited at 10 μg/ml 12G5. This indicates that rat sequences outside E2 must influence the recognition by 12G5 and its capacity to inhibit infection. This would be consistent with a reduced detection of chimera M by 12G5 in FACS being due to a reduced affinity, rather than surface expression. Thus, although E2 must contain part of the 12G5 conformational epitope, other region(s) of the receptor are likely to influence its affinity.

The CXCR4 ligand, SDF-1α, blocked HIV-$2_{ROD/A}$ and HIV-$2_{ROD/B}$ entry in a dose-dependent manner on CCC cells expressing CXCR4 (Fig. 3*b*). Unlike 12G5, SDF-1α inhibition of HIV-2_{ROD} infection was equally efficient on cells expressing chimeric receptor M (human E2 in rat CXCR4) or human CXCR4. This shows that rat sequences outside E2 are not detrimental to human SDF-1α binding. The CC chemokine RANTES, which does not bind to CXCR4, had no effect on CD4-independent CXCR4-mediated HIV-2_{ROD} infection. These results confirm that CD4-independent entry is specifically mediated by CXCR4 and emphasize the essential role played by the second extracellular loop of CXCR4. We also recently showed that the N-terminal 23 aa of CXCR4 were involved in HIV-2_{ROD} infection of $CD4^-$ cells (Potempa *et al.*, 1997). Therefore both E2 and N-terminal sequences are likely to be involved in the HIV-2 Env–CXCR4 interaction.

Mutations in ROD/B Env (SU and TM) responsible for its CD4-independent phenotype have been determined (Reeves & Schulz, 1997). One amino acid change from the ROD/A genotype resides in the TM subunit, two flank the V4 loop and the fourth change, which enhances a minimal CD4-independent phenotype, is in the V3 loop. It has been proposed that the initial CD4 binding, which occurs in CD4-dependent entry, induces conformational changes in the Env proteins, exposing a region involved in binding to a second receptor or coreceptor (reviewed in Moore *et al.*, 1993, 1997). The mutations in ROD/B may alter the Env conformation so that the binding site for CXCR4 is exposed in the absence of CD4, and the activation threshold required for HIV-2-induced fusion of $CD4^-$ cells is reduced (Reeves & Schulz, 1997).

Our results show that the interaction of HIV-$2_{ROD/B}$ with CXCR4 is similar to that of CD4-dependent HIV-1 isolates, suggesting that the Env–coreceptor interaction is conserved between CD4-dependent and -independent viruses, as well as between HIV-1 and HIV-2.

Although primary CD4-independent HIV-1 or HIV-2

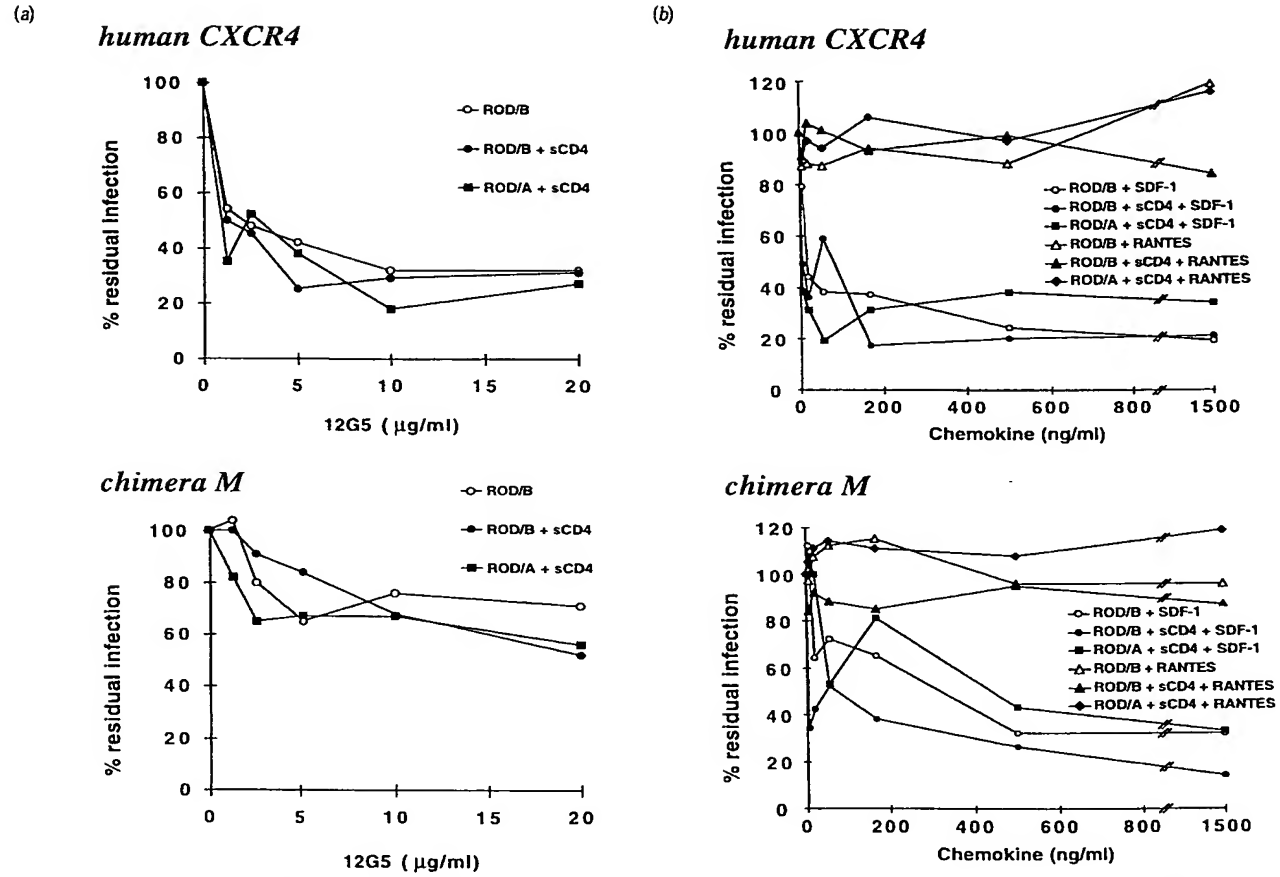

Fig. 3. Inhibition of HIV-$2_{ROD/B}$ infection of CCC cells expressing human CXCR4 or human E2-containing receptor (receptor M) by (*a*) anti-CXCR4 12G5 MAb and (*b*) CXCR4 natural ligand, SDF-1α. Transfected cells were incubated with increasing concentration of monoclonal antibodies or chemokines prior (40 min) and during infection (3 h). Results are from a representative experiment and infectivity is expressed as a percentage of the maximum infection (calculated in f.f.u./ml) observed without antibodies or chemokines.

isolates have not yet been identified, there is evidence of infection of $CD4^-$ cells in HIV-infected humans (Cohen, 1995; Housset *et al.*, 1993; Lee *et al.*, 1997; Livingstone *et al.*, 1996; Wiley *et al.*, 1986). It is, however, unclear if infection of $CD4^-$ cells influences virus pathogenesis. Nevertheless, any therapy targeted at HIV entry should take into account the possibility of CD4-independent infection *in vivo*, since the potential is obvious from the emergence of such strains *in vitro*. Our studies into the mechanism of CD4-independent infection will therefore assist assessment of the suitability of such therapeutics as they are developed.

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Methodology article

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Fully automated synthesis of (phospho)peptide arrays in microtiter plate wells provides efficient access to protein tyrosine kinase characterization

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Abstract

Background: Synthetic peptides have played a useful role in studies of protein kinase substrates and interaction domains. Synthetic peptide arrays and libraries, in particular, have accelerated the process. Several factors have hindered or limited the applicability of various techniques, such as the need for deconvolution of combinatorial libraries, the inability or impracticality of achieving full automation using two-dimensional or pin solid phases, the lack of convenient interfacing with standard analytical platforms, or the difficulty of compartmentalization of a planar surface when contact between assay components needs to be avoided. This paper describes a process for synthesis of peptides and phosphopeptides on microtiter plate wells that overcomes previous limitations and demonstrates utility in determination of the epitope of an autophosphorylation site phospho-motif antibody and utility in substrate utilization assays of the protein tyrosine kinase, $p60^{c\text{-}src}$.

Results: The overall reproducibility of phospho-peptide synthesis and multiplexed EGF receptor (EGFR) autophosphorylation site (pY1173) antibody ELISA (9H2) was within 5.5 to 8.0%. Mass spectrometric analyses of the released (phospho)peptides showed homogeneous peaks of the expected molecular weights. An overlapping peptide array of the complete EGFR cytoplasmic sequence revealed a high redundancy of 9H2 reactive sites. The eight reactive phospopeptides were structurally related and interestingly, the most conserved antibody reactive peptide motif coincided with a subset of other known EGFR autophosphorylation and SH2 binding motifs and an EGFR optimal substrate motif. Finally, peptides based on known substrate specificities of c-src and related enzymes were synthesized in microtiter plate array format and were phosphorylated by c-Src with the predicted specificities. The level of phosphorylation was proportional to c-Src concentration with sensitivities below 0.1 Units of enzyme.

Conclusions: The ability of this method to interface with various robotics and instrumentation is highly flexible since the microtiter plate is an industry standard. It is highly scalable by increasing the surface area within the well or the number of wells and does not require specialized robotics. The microtiter plate array system is well suited to the study of protein kinase substrates, antigens, binding molecules, and inhibitors since these all can be quantitatively studied at a single uniform, reproducible interface.

Background

Phosphorylation and dephosphorylation of proteins are major mechanisms mediating signal transduction throughout the cell and are intimately involved in the regulation of cell growth, physiology, differentiation, and death. Phosphorylation is accomplished by means of kinases which when stimulated by an afferent signal transmit the signal via phosphate transfer to the next site in a pathway. In some cases phosphoprotein-protein interactions take place that modulate signal transduction, e.g. by revealing previously sequestered phosphoacceptor sites in one or both of the interacting proteins, thus creating branch points in pathways. Critical questions exist regarding the identification of the *true in vivo* substrates of kinases, identification of phosphotyrosine interaction domains, and mapping the radiation of these protein interactions throughout extremely complex networks. Clearly new technologies capable of accelerating the processes for defining the interactions between kinases and their substrates and modulators would be of great value.

Two highly productive approaches have been the determination of optimal substrate motifs favored by individual kinases, by various combinatorial peptide library approaches and, the use of antibodies to study phosphorylated peptide motifs (reviewed in [1,2]). Synthetic peptides have played a long and useful role in characterizing kinase substrate sequences, particularly for the ser/thr family, which is now seen to consist of a few distinct category types, basophilic, acidophilic and proline directed. Protein tyrosine kinases, on the other hand, are less well defined by their natural substrates but make more use of docking intermediaries to perform the task of substrate recognition. Nevertheless, optimal substrates have been found which can then aid in the search for the identity of natural or *in vivo* targets and inhibitors of the kinase [3,4]. While capable of assessing mixtures of very large numbers of random peptides, combinatorial methods require deconvolution strategies, which can be time-consuming, and technically demanding.

A second search strategy for functional peptides employs arrays of spatially addressable peptides that can be tested *in situ*, accelerating the deconvolution process when the number of combinations is, or becomes more limited. Peptide arrays capable of displaying diverse functions including kinase substrate activity have been successfully produced by two methods: *in situ* synthesis on planar membranes or arrays of pins [5-10], or attachment of preformed peptides as performed in a variety of microarray printing procedures. While the existing synthetic methods are capable of producing large numbers of peptides in good purity, none are fully automatable. They require manual intervention between each synthesis cycle and thus are not totally automatable. Peptide synthesis in microtiter plate wells would allow the use of fully automated robotic handlers. Further, arrays of peptides produced in a microtiter plate format, which is an industry standard for numerous types of high throughput analytical procedures, could also be tested in automated multiplex fashion.

We present data here demonstrating the applicability of an automated system for peptide design and synthesis in microtiter plates to the production of peptides and phosphopeptides. We further demonstrate the capability of these peptide arrays to be recognized correctly by specific phospho-motif antisera and to serve as kinase substrates.

Results and discussion

(Phospho)peptide synthesis method

Previous work led to the development of a system for the automated synthesis of peptide arrays on the inner surfaces microtiter plate wells [11-13], (Figure 1). However, direct characterization of the synthesized peptides did not become realizable until the recent availability of modern high-sensitivity mass spectrometers. In this method polymethylpentene (TPX) microtiter plates are activated by oxidation with nitric acid, and made functional for peptide synthesis by condensation with poly(D-lysine) (n = 100), which serves as a free-floating support or polymeric handle containing an extended array of amino groups. Each lysine side-chain then serves as an initiation point for synthesis. The synthesized peptides are extended from the well surface by their attachment through their peptide carboxyl-termini, on a molecular tether of average estimated length of 50 lysine subunits. This simply assumes that each polylysine molecule is attached to the surface through one bond at its midpoint. It is expected that multiple attachment bonds between the surface and a single polylysine chain can also form but would be minimized by the 2000-fold molar excess of polylysine over attachment capacity used during the polylysine coating step. Model experiments have shown that the polylysine helical structure would be maintained in solution [14,15] for most, if not all cases. If multiple attachment bonds were formed then distal ends would prefer the helical form while the sequences between attachment points should also prefer the helical form up to the limits imposed by torsional constraints, and depending on the closeness of the attachment positions. Thus, the peptides are well positioned spatially, to interact with a variety of macromolecules such as antibodies and structures as large as a cell surface. The lengths and sequences of the peptides are programmable; the total elapsed cycle time to extend each peptide by one residue for all 96 wells is 1 hour. The process has been optimized with respect to activation conditions, length and composition of the polymeric tether and conditions for the handling and storage of the prediluted amino acid derivatives and condensing agents.

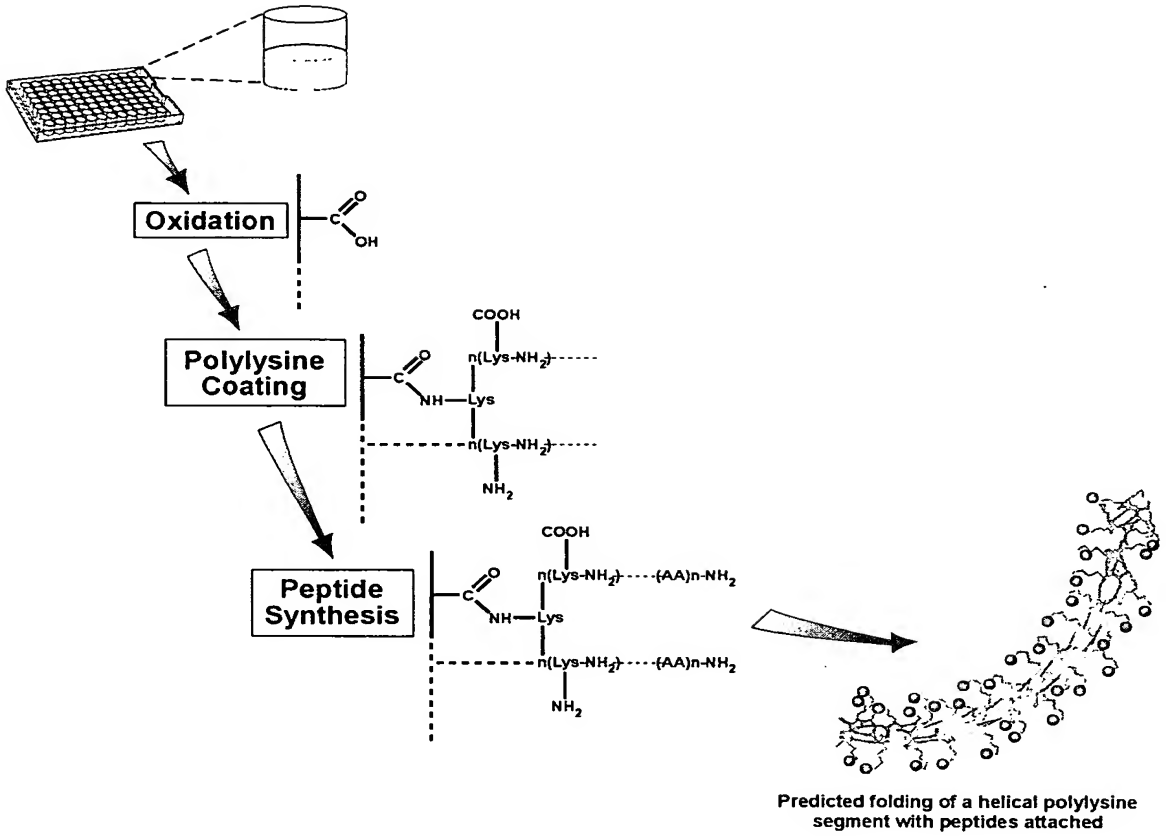

Figure 1
Microtiter plate peptide array chemistry Costar TPX microtiter plate strip well surfaces were oxidized with nitric acid, coated with polylysine and peptide synthesis is performed as described in Methods. Attachment of the polylysine chains to the wells and initiation of peptide synthesis takes place through the lysine ε-amino groups. The predicted polylysine helical segment shown is stabilized primarily through backbone H-bonding and synthesized peptides are depicted by red spheres.

Stabilities exceeding two years have been achieved for all reagents used. The capability of these peptides to be recognized by antibodies, leading to the identification of sequences and structures of the immunoreactive domains of viral proteins and biological response modifiers has been previously been demonstrated [11-13].

Reproducibility and quality of synthetic peptides
To evaluate the fidelity and reproducibility of peptide syntheses and ability of the synthetic peptide arrays to serve as specific targets in sequence defined molecular affinity interactions, a model system was chosen to provide known test parameters. The EGF receptor system was selected since it provided commercially available monoclonal antibodies with documented specificity for an activation state associated autophosphorylation site (pY1173) and known sequence (NAEpYLRV). Testing was begun with the monoclonal antibody 9H2 produced against a peptide containing the NAEpYLRV sequence. An array of alternating NAEYLRV and NAE(pY)LRV peptides was prepared in a microtiter plate consisting of 12 8-well strips. Monoclonal 9H2 antibody ELISA was performed using three of the strips from the middle section of the plate. It was found that the antibody reacted strongly with the peptide wells containing phosphorylated tyrosine but not with the non-phosphorylated peptide wells or control

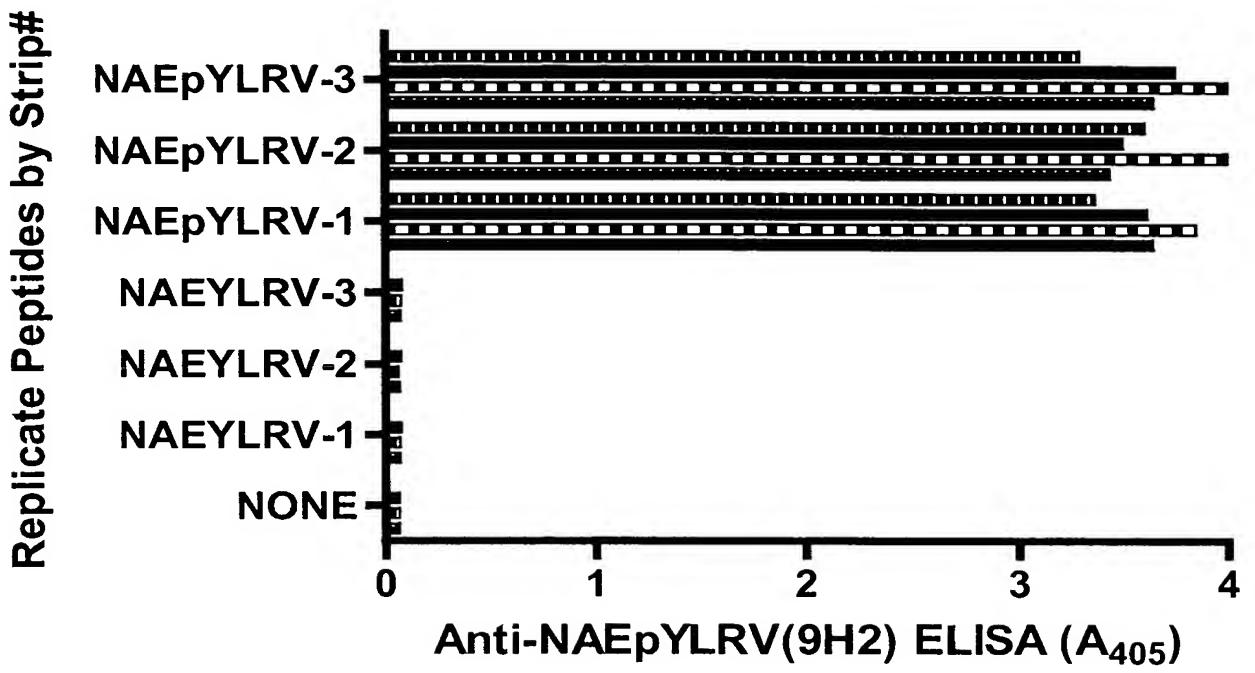

Figure 2
Reproducibility and specificity of multiplexed EGFR peptide synthesis and ELISA testing Peptides were synthesized as microtiter plate array strips using either tyrosine or phosphotyrosine. Three replicate strips were used, each with no peptide in row A and alternating phosphotyrosine and tyrosine peptides in rows B to H. The strips were tested for reactivity with clone 9H2 antibody as described in Methods. ELISA results (x-axis) are plotted against tyrosine or phosphotyrosine replicates for each strip (y-axis). Standard deviations varied between 5% and 10% of the means. Data are displayed without any correction.

wells without peptide (Figure 2). For the three strips ELISA means and (standard deviations) for the phosphotyrosine peptides were 3.62(0.20), 3.64(0.25), 3.67(030) and for the tyrosine peptides were 0.071(0.003), 0.068(0.007), 0.076(0.004). For wells containing no peptide, the values for all three strips were 0.063(0.0006). The coefficients of variation for all replicate sets ranged between 5% and 8%.

For evaluation of the fidelity and authenticity of phosphorylated and non-phosphorylated peptide products wells containing NAEYLRV and NAEpYLRV were prepared as before except that a cleavable linker was added to the polylysine matrix before peptide synthesis. The synthesized peptides were then cleaved from the surface with TFA using conditions under which the protecting groups were removed from the amino acid side-chains but not from tyrosine phosphates. When the released peptide products were concentrated and analyzed by MALDI-TOF-TOF mass spectrometry it was found that both peptides yielded essentially monodisperse m/z of the predicted molecular weights, 1036.56 for NAEYLRV and 1170.62 for NAEpYLRV (Figure 3A and 3B, respectively; [bis(dimethylamino)phosphono]-tyrosine species shown). These data demonstrate high coupling efficiency at each step of synthesis and stability of the activated amino acids throughout the process.

Redundancy of an autophosphorylation site antibody epitope in the EGFR cytoplasmic domain

Since many of the twenty tyrosines found in the EGFR cytoplasmic domain are known to serve as substrate or SH2 binding site for other tyrosine kinases, it was decided to test the specificity of the 9H2 antibody. To examine this question, overlapping peptide arrays covering the transmembrane and cytoplasmic domains of the EGF receptor were prepared. One array contained only

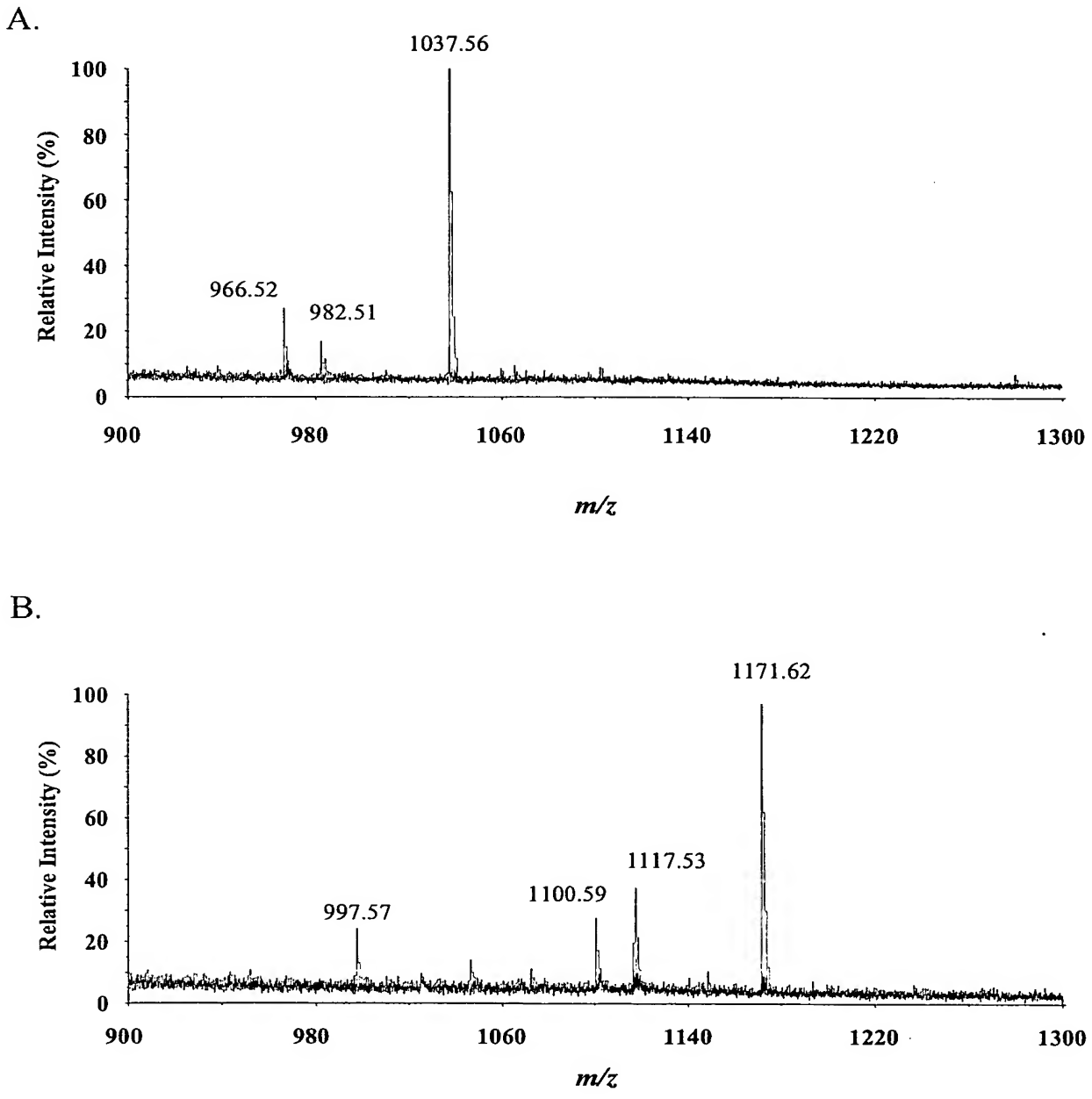

Figure 3
Mass spectrometric characterization of EGFR phosphopeptides produced in microtiter plate arrays Panel A illustrates the mass spectrum of the NAEYLRV derivative ($[M+H]^{1+}$ ion at *m/z* 1037.56) and panel B for the NAEpYLRV derivative ($[M+H]^{1+}$ ion at *m/z* 1170.62). Peptides were released by incubation for 3 hours in TFA-H_2O-triisopropylsilane, concentrated *in vacuo* and reconstituted for MALDI-TOF-TOF analysis. Under these conditions the bis(dimethylamino) phosphate protecting groups of phosphotyrosine were preserved and mass spectra were improved.

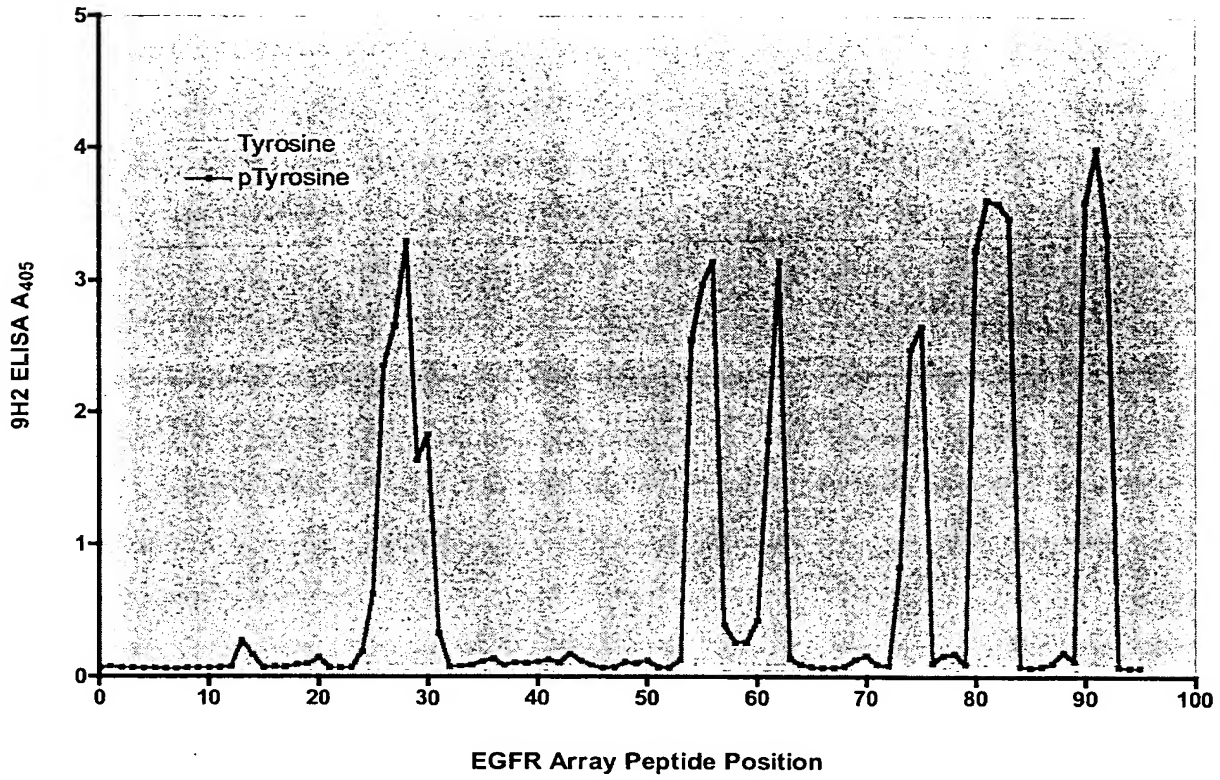

Figure 4
Distribution of 9H2 phosphotyrosine epitopes in EGFR Overlapping peptide arrays were made and acetylated in microtiter plates using human EGF receptor residues 622–1186 (Swissprot database locus P0053 with leader sequence removed). Each array contained exclusively tyrosine or phosphotyrosine peptides and was composed of 92 peptides each of which was 21 amino acids long and offset from its neighbor by 6 amino acids. Array peptide wells were reacted in multiplexed ELISA format with antibody 9H2 as described in Methods. Data are plotted without any corrections. Peptide sequences and raw data are listed [see Additional file 1].

phosphotyrosine and the other only tyrosine. The arrays consisted of 92 peptides, each of which was 21 amino acids in length and overlapped by 15 amino acids. In the EGFR sequence arrayed, there are 20 unique occurrences of tyrosine-containing peptide sequences. In the array, each such sequence appears three to four times in progressively overlapping fashion. Clone 9H2 antibody showed high reactivity (from 9 to 57 times background) with eight out of the twenty phosphotyrosine-containing sequences (Figure 4, also [see Additional file 1]). In general, each reactive peak was associated with three to four appearances of the identifiable tyrosine as its position moved progressively along the overlapping peptide sequences, suggesting consistent reliable synthesis throughout the synthetic process. None of the non-phosphorylated tyrosine-containing peptides showed any comparable reactivity with the monoclonal antibody 9H2 although a barely detectable level of antibody reactivity with all peptides containing tyrosine could be seen. There was no detectable reactivity against peptides not containing tyrosine [see Additional file 1]. Steric hindrance by the attachment matrix did not appear to be a significant problem in the recognition of reactive peptide sequences separated by just one, and two amino acids from the peptide carboxyl terminus ([see Additional file 1], array positions 81, 91)

To assess the significance of these primary cross-reactivities a consensus table was constructed from 9H2 antibody

Tyrosine Sequence Position	Phosphotyrosine Sequence	Derived from Peptide Array Position* (active..fragment)	ELISA Result*	Foot-Notes
777	...GCLLD-pY-VREHK...	26, 27	+	
789	...IGS-pY-LLWC...	(27), 28, 29	+	
803	...AKGM-pY-LEDRR...	(29), 30, 31	+	
954	...RDPR-pY-LVIG...	55, 56, (57)	+	
992	...VDADE-pY-LIP...	(61), 62, (63)	+	1, 2
1068	...LPVPE-pY-I...V...	74, 75, (76)	+	1, 2
1114	...VGPE-pY-L...V...	(81), 82, 83, (84)	+	
1173	...AEAE-pY-LRVAP...	(91), 92, 93	+	1, 4
703	...AFGV-pY-KGLWI...	13, 14	-	
730	...ILDEA-pY-VMASV...	20	-	
845	...AEEKE-pY-HAEGG...	37, 38	-	5
867	...ILHRI-pY-THSD...	41, 42	-	
875	...SDVWS-pY-GV...VW...	42, 43	-	
891	...FGSKP-pY-DGIPA...	45, 46	-	
920	...C...IDV-pY-MIMVK...	50	-	
974	...SDSNF-pY-RALMD...	59	-	1, 6
1045	...SFLQR-pY-S...DP...	70, 71	-	1, 7
1086	...VQXPV-pY-HNQPL...	77, 78	-	1, 3
1101	...SRDPH-pY-QDPHS...	80, 81	-	5
1148	...LDXPD-pY-Q...DFF...	88	-	1, 8

Amino acids are coded for acidic, basic, hydrophilic, and hydrophobic as red, blue, green and brown.

Figure 5

Pattern of recognition of arrayed EGFR peptides by autophosphorylation site antibody (9H2) Tyrosine sequence positions correspond to human EGF receptor residue positions (Swissprot P0053, leader sequence removed). Amino acids are color coded for acidic, basic, hydrophilic, and hydrophobic as red, blue, green and brown. *. For (phospho)peptide array compositions and quantitative results [see Additional file 1]. Footnotes: 1. autophosphorylation site [26-28], 2. PLC-gamma SH2 domain binding[29], 3. GRB2/SH2 domain binding [30], 4. Shc, SHP1, PLC-gamma SH2 domain binding [31, 32], 5. Src phosphorylation site [33-35],6. AP2 [36, 37], 7. Cbl, SH2 domain binding [38-40], 8. Shc SH2 domain binding [31, 32, 41].

reactive and non-reactive sequences (Figure 5). A strong preference for hydrophobic amino acids in the Y+1 position is readily apparent with leucine the most preferred appearing in 6 of 8 peptides, followed by isoleucine and valine, both appearing once. At the Y-1 position glutamic acid was the most preferred, appearing in 4 of 8 peptides, followed by arginine, asparagine, aspartic acid, and glutamine with one appearance each. Thus the preference appears to be primarily for glutamic acid but other hydrophilic amino acids were also accepted. There did not

appear to be any discernable pattern of preference at the remaining peptide positions. The least common denominator among positive peptides therefore appears to be E/(R, N, D, Q)-pY- L/(V, I), or E-pY-L in its predominant form. Peptide sequence # 84 [see Additional file 1] which lacked an acidic or hydrophilic amino acid at the pY-1 position was still strongly reactive demonstrating the strong contribution of a hydrophobic amino acid in the Y+1 position. However, since the peptide at position Y730 containing A(pY)V was not reactive ([see Additional file 1], Figure 5), 9H2 binding appears to involve more than the pY-hydrophobic sequence alone.

Three of the 9H2 cross-reactive phosphotyrosines are clustered within the C-terminal end of the receptor (992–1173), are known autophosphorylation sites and are known to be recognized by proteins containing Group III SH2 binding domains [16] (e.g. p85, phospholipase $C_{\gamma 1}$, the tyrosine phosphatases, Figure 5), that similarly recognize phosphotyrosines with hydrophobic amino acids at the Y+1 position. Since some of the 9H2 cross-reactive epitopes are not associated with phosphoacceptor activity it suggests that the phosphoacceptor site specificities are more stringently controlled than the 9H2 epitope or that phosphoacceptor activity simply has not been demonstrated yet. A similarity in the processes for recognition of specificity determinants within the deduced epitope of the 9H2 autophosphorylation site antibody and optimal substrate motif found for the EGF receptor [16-18], EEEEY-FELV, may also exist.

Twelve of the twenty tyrosines present in the EGFR cytoplasmic domain were not reactive with 9H2. None of the twelve conformed to the deduced 9H2-epitope motif, although six out of 12 were involved with other aspects of kinase signal transduction (Figure 5). As confirmation that this was not a result of failure to incorporate phosphotyrosine in the negative peptides a plate array containing all of the EGFR phosphotyrosine peptides was constructed and tested against a group of commercial phosphotyrosine antibodies prepared in various ways. These results are shown in Figure 6. Antibodies 4G10, AB8076, and PT101L were prepared using immunogens which were chemically modified or haptenized and not known to be sequence restricted and all reacted strongly with all of the EGFR phosphopeptides. Antibody RDI-egfract-1 was prepared against the activated EGF receptor and known to be activation specific but not known to be active against linear peptides. These results add support to the specificity of the epitope deduced above and contribute additional useful information and reagents, namely confirmation of the pan-specific nature of the phosphotyrosine antibodies as used in the microtiter plate peptide array system

Tyrosine kinase activity on synthetic peptide substrates in microtiter plate arrays

To characterize the quantitative aspects of substrate phosphorylation by c-Src kinase, a panel of peptides based on known substrate specificities of c-src and related enzymes were synthesized in microtiter plate wells. Each peptide extended from the polylysine backbone by an ε-amino side-chain and by an additional C-terminal Cys unit. Thus, all of the peptides were equally available to the enzyme, with reduced steric hindrance and a uniform presentation. Subsequently, 90 μL of reaction buffer containing varying amounts of c-Src kinase were incubated for 20 min at 30°, according to the manufacturer's instructions. The wells were then washed with distilled water and assayed for the presence of phosphotyrosine by ELISA using a mixture of the broadly cross-reacting phosphotyrosine antibodies previously described in Figure 6. The peptide substrates EEIYGEFF [17] (Src,1) and YIYGSFK [19] (Src,2) have been shown separately by somewhat different combinatorial methods to have relatively potent activity for protein tyrosine kinases and are shown to be reactive here as well (Figure 7). (Src, 1) was phosphorylated to a greater extent than (Src, 2) and showed no decrease of reactivity even at 0.1 Unit of enzyme, the lowest concentration used. The (Src, 1) variant peptides were chosen so that validation could be made by direct comparison with the highly oriented peptide chip system recently described [20]. The (Src, 1) variant peptide (-E) made by truncation of the N-terminus still showed good reactivity, although much lower than the longer (Src,1) peptide, and the (-Y) peptide in which tyrosine was exchanged for phenylalanine showed no reactivity as expected and required. The slight c-ABL tyrosine kinase substrate peptide IYAAPKKK [17] reactivity at the highest c-Src input and negative protein kinase A substrate peptide LRRASLGC [21,22] activity are consistent with the level of cross-reactivities expected between familial and nonfamilial kinases. Three of the peptide substrates showed dose-response characteristics conforming, by nonlinear regression, to a one site binding model for (-E), (Src, 2), and (ABL), with R^2 equal to 0.98, 0.99, and 0.92. Activity of the most reactive peptide, (Src, 1) was greater than expected based on quantity of enzyme used in previously published work [23,24] and was at least 30 times more reactive than its truncated form (-E) [20]. By extrapolation, c-Src activity would be detectable at concentrations as low as 0.01 Units of enzyme using the (Src, 1) substrate.

Conclusions

The method for production of synthetic peptide solid-phase arrays in microtiter plates described here is capable of making high quality peptides, as seen by mass spectrometry of the released unfractionated products. The peptide synthesis method is completely automated and has

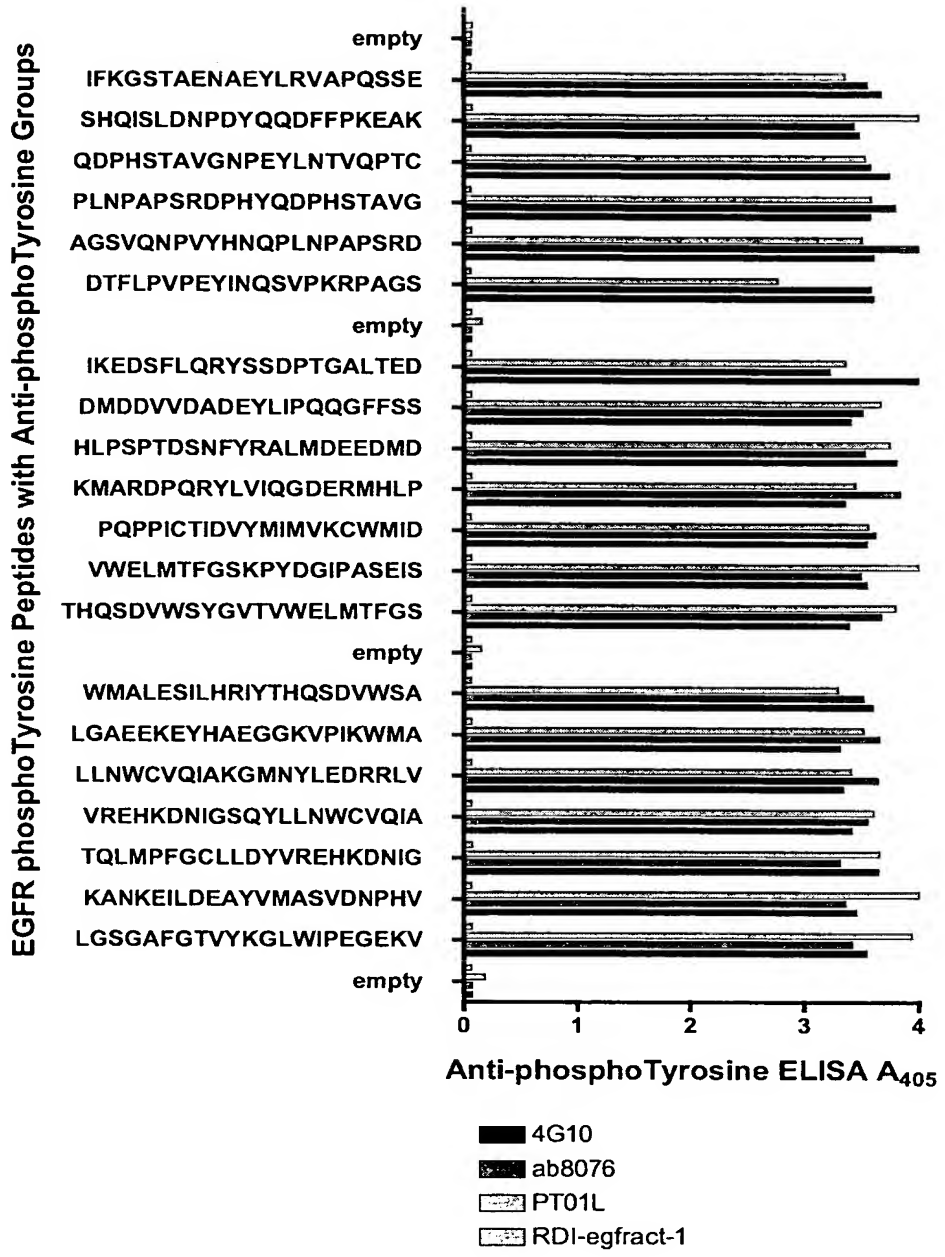

Figure 6

Reactivity of all EGFR phosphotyrosine peptides with a panel of pan-specific phosphotyrosine monoclonal antibodies Phosphotyrosine peptide sequences selected from the arrays used in Figure 4 [see Additional file 1] were synthesized in a new microtiter plate array and are identified on the y-axis. Each peptide and an empty control well were tested with each of the four antisera identified in the Figure legend, 4G10, ab8076, PT01L, and RDI-egfract-1 by standard ELISA as described in Methods. Data are plotted without any corrections

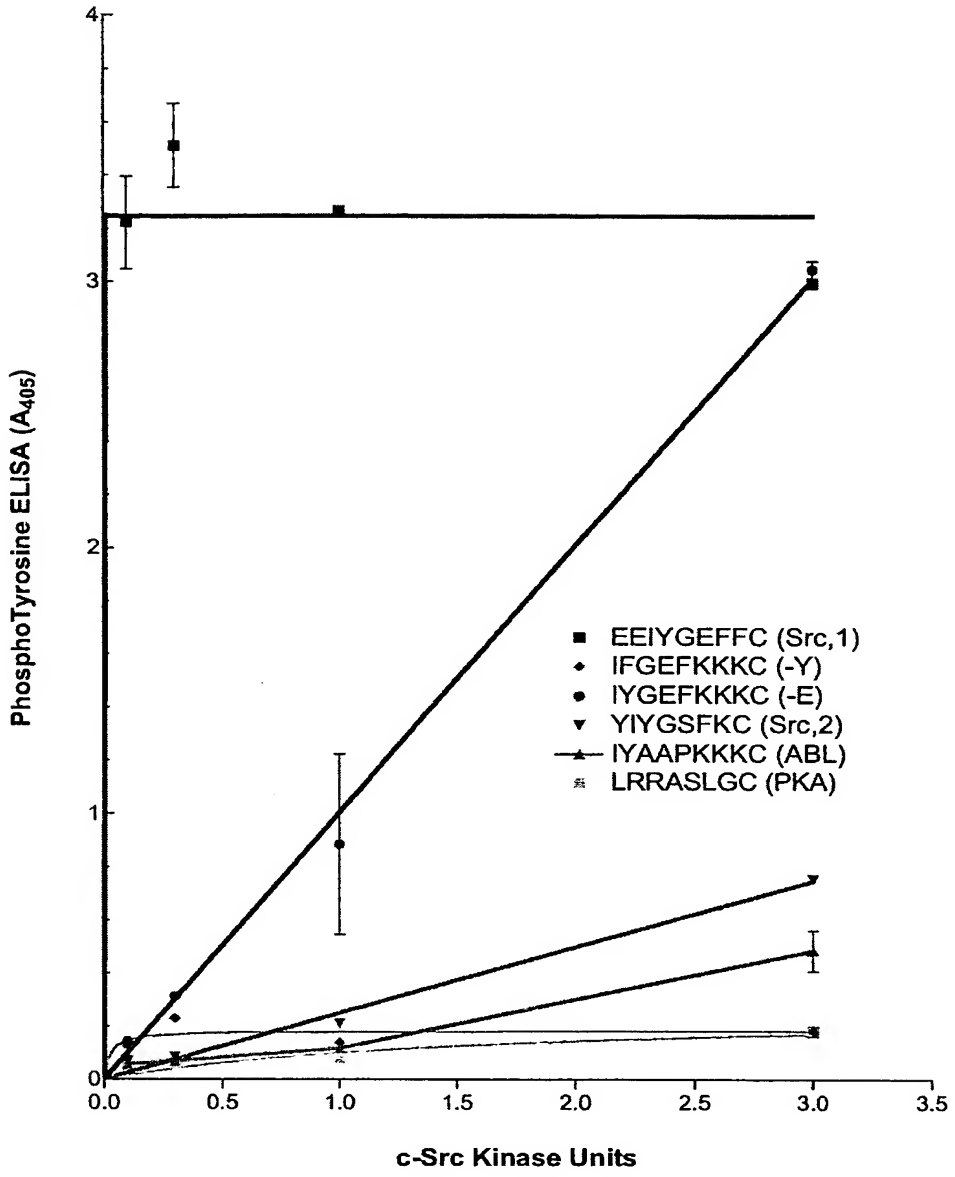

Figure 7

Microtiter plate array peptides serve as substrates for $p60^{c\text{-}src}$ with specificity and concentration dependence

Eight-well strips bearing six substrate peptide wells and two control wells were robotically synthesized *ab initio*, in microtiter plate format. All of the peptide substrates were initiated with Cys(Acm) to provide additional extension from the polylysine backbone and a common attachment site. The Cys-SH protecting group (Acm) remains in place and all peptides were acetylated at the amino terminus. Duplicate substrate strips were reacted with each 90-μL dilution of c-Src enzyme containing 3, 1, 0.3, and 0.1 Units of enzyme. The wells were reacted for 20' at 30°, washed with distilled water and assayed by antibody ELISA for the presence of phosphotyrosine using a mixture of antibodies described in Figure 5. Nonlinear regression plots were computed in Prism and error bars represent the means ± 1 SD, which ranged between 3% and 7% of mean values. Iterations for (ABL) did not converge. All data points were corrected by subtraction of values obtained from wells with no peptide.

been greatly simplified by the use of standard automated liquid handlers and the use of activated amino acid solutions that may be prepared and stored in advance and added just once at the beginning of the synthetic process. In the present demonstration, 96 well microtiter plates were used; but, 384, 1536, or containers of any well density compatible with the solvents and liquid handler may be used. The solid phase peptide arrays produced on a polylysine backbone were found to be of very high density, provide very low levels of nonspecific binding and steric hindrance, and participate effectively in a variety of biochemical reactions.

The strategy described here for the preparation of solid phase synthetic peptide arrays in microtiter plate wells for use in multiplexed assays offers many advantages in the study of protein kinases, particularly in a research environment. In a research environment combined cycles of hypothesis generation and testing, with assay flexibility, speed, quantitative accuracy and precision are of greater concern than in large scale screening applications of large numbers using limited, previously selected variables. The industry standard microtiter plate format ensures compatibility with a vast number of assay platforms and the polylysine backbone with its extended three-dimensional display provides a highly efficient, sterically unhindered, and extremely low background display of the peptide products.

Using 96-member peptide arrays of 21-mers created in less than 24 hours, we have shown that the peptide array synthesis provided a highly reproducible model for a tyrosine peptide, EGFR Y1173 and its phosphorylated counterpart. Using a monoclonal antibody prepared against a synthetic peptide representation of the Y1173 EGF receptor autophosphorylation site, we have provided evidence that, unexpectedly, the deduced epitope, E/H_L-pY-L/H_B (where H_L- is hydrophilic and H_B is hydrophobic) is highly redundant within the cytoplasmic domain. Three of the eight antibody reactive sites have been previously identified as autophosphorylation sites and are recognized by Group III SH2 domain proteins (Figure 5) that have similar specificity patterns. Furthermore, the EGFR substrate sequence EEEEYFELV, derived by combinatorial peptide optimization [17], resembles the E-Y-(hydrophobic) motif found in these studies. Thus there is a consistent linkage between a subset of EGFR phosphotyrosine sequences recognized by the 9H2 antibody, a subset of sequences autophosphorylated by EGFR kinase, and EGFR autophosphorylation sites recognized by the Group III SH2 domain. There is a clear parallel between the 9H2 peptide epitope and the peptide substrate specificity of the EGFR catalytic activity [16]. Songyang has further suggested that the catalytic and SH2 domains of PTKs may have converged to recognize similar sequences. So, questions regarding which site (or sites) the antibody actually recognizes on stimulated EGF receptor molecules and what other parallels might exist between 9H2 binding, SH2 binding, and catalytic substrate selection become of interest. At the cell protein level, 9H2 is specific by Western blot for the stimulated EGF receptor. Furthermore, there are preliminary data (Saxinger, unpublished) suggesting that PDGF peptide sequences conforming to the 9H2 binding sequence of (hydrophilic)-pY-(hydrophobic) deduced from EGFR phosphotyrosine peptides, appear to be recognized differently by 9H2. While nine of the twenty-seven PDGFR phosphotyrosine sequences satisfied the (hydrophilic)-pY-(hydrophobic) sequence definition, and could be expected to be as reactive as those in EGFR, only one showed comparable reactivity. Thus, the binding determinants of 9H2 are more complex than the simple epitope deduced from Figure 5. An intriguing possibility is that the 9H2 epitope may be a fairly simple one but that its appearance, or access to it has been limited or distorted in specific ways by spatially adjacent sequences or structures that have evolved to create opportunities for exploitation in biologically specific processes.

We have also successfully demonstrated the use of microtiter plate peptide arrays in faithfully reproducing the known substrate phosphorylation specificities of c-Src protein kinase. In these studies a broadly reactive phosphotyrosine antibody ELISA detected phosphorylation. Although a mixture of antibodies that were not known to be sequence restricted was used, the possibility exists that some tyrosine-containing peptides could become phosphorylated and be recognized less well than others. Therefore in studies where the need for precise quantitation outweighs the convenience and safety considerations of ELISA, incorporation of radioisotopic phosphate would provide an alternative. The microtiter plate format with reactants bound to the well surface would provide a well-contained and safe vehicle for washing and subsequent measurement of radioactivity.

Thus, the microtiter plate array system is well suited to the study of protein kinase substrates, antigens, related binding molecules, and inhibitors since these all can be quantitatively studied at a single uniform, reproducible interface. For applications requiring larger numbers of solid phase peptides, the synthetic process can easily be transferred to more powerful workstations such as the Biomek FX platform in which many plates with higher well densities can be synthesized simultaneously and conventional particle based substrates for peptide synthesis can be manipulated using filter plates or magnetic devices available for this system. Moreover, the current capacity of approximately 150–380 pMoles/well can be considered large enough for preparative or analytical applications coupled with mass spectrometric analyses, such as affin-

ity-based proteomic screening for ligand-protein interactions. In this and other applications, such as assessment of protease activity where strict isolation of adjacent components is required, microtiter plate wells enjoy a significant mechanical advantage over two-dimensional spotting or other synthesis methods.

Methods

Materials

Solvents and reagents for peptide synthesis were obtained as synthesizer grade from Applied Biosystems (Foster City, CA). Specifically, these were N-methylpyrollidone (NMP), 1 M N-Hydroxybenzotriazole (HOBT) in NMP, 1 M dicyclohexylcarbodiimide (DCC) in NMP, diisopropylethylamine (DIPEA), acetic anhydride, and trifluoroacetic acid (TFA). NMP from this vendor was consistently free of basic impurities and was stored over Molecular Sieve (4A) after opening. NMP solutions were stored at -20C and allowed to attain room temperature before opening. FMOC-protected α-amino acids were purchased from Penninsula Laboratories (San Carlos, CA) and side-chain substitutions were, Asn(Trt), Asp(OtBu), Cys(Acm) or Cys(Trt), Gln(Trt), Glu(OtBu), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu) and Tyr(tBu). FMOC-Arg(Pbf), FMOC-[bis(dimethylamino)phosphono))-tyrosine] [25], and FMOC Rink amide (linker p- [(R,S)-a-[1-(9H-Fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid) were purchased from NovaBiochem. Phenol, thioanisole, ethanedithiol (EDT), triisopropylsilane (TIPS), and carbonyldiimidazole (CDI) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Poly(D-Lys•HBr) (dp 100) and Poly(L-Lys•HBr) (dp 100) were obtained from Sigma-Aldrich (St. Louis, MO). TPX (polymethylpentene) 8-well strips, non-sterile, non-tissue culture treated, were obtained from Costar on special order (Cambridge, MA).

(Phospho)Peptide synthesis

Automated preparation of solid phase synthetic peptide arrays in microtiter plates was performed as described previously [13] (Saxinger, US Patent 6031074, Feb. 29, 2000). Carboxyl functional groups are formed on the hydrocarbon surface of Costar TPX microtiter plate strip wells by oxidation with 70% nitric acid for two hours at 65°C or for two weeks at room temperature. Polylysine chains are then attached to the surface by condensation using 0.05 M carbonyldiimidazole in NMP for 30' at 20°C. Poly(L-Lys•Hbr) (PLL) or poly(D-Lys•Hbr) (PDL) (1 mg/ml in 90% NMP-10% water) was neutralized with DIPEA and reacted with the CDI-activated surface for 1 hour at 20°C and followed by 4°C overnight. Plates were rinsed twice with water and twice with methanol, air-dried, wrapped in plastic cling-wrap and stored at room temperature. Peptide synthesis next takes place by the sequential addition of Nα-FMOC amino acids activated with DCC/HOBT as in conventional peptide synthesis with the growing peptide chain covalently attached to the polylysine chain through its carboxyl terminus. Stock solutions of FMOC amino acids were prepared in advance by dissolving 6 mmoles of each in 12 mL of NMP containing a 10% molar excess of HOBT, and stored at -20°C. The solutions could then be used repetitively for at least three months. Prior to peptide synthesis, the required amounts of amino acid were diluted to 0.1 M in NMP, mixed with an equimolar volume of 0.1 M DCC in NMP and allowed to react for thirty min in 2 mL cryovials. The 20 mixtures were then placed in a 24 tube rack on the Biomek 1000 workstation tablet for use during subsequent peptide synthesis cycles. Synthesis automation is achieved through software that receives input designating the peptide desired in each of the 96 microtiter plate wells and creates output files to indicate the quantity of reagents needed and a set of Beckman Biomek 1000 arrays.bio files, each member of the set directing the distribution of amino acids in one of the sequential chain extension cycles. An automated repetitive set of reagent transfers and washing steps is then recycled using a new arrays.bio file for each cyle until the peptide synthesis is complete. After synthesis the peptides are usually capped by reaction with acetic anhydride (1 mL in 10 mL of NMP containing 0.1 mL of DIPEA per plate) before side-chain deprotection and testing, in multiplex fashion, for reactivity. Side chain protecting groups were generally removed with: 10 mL of TFA +0.75 g of crystalline phenol + 0.5 mL of purified water + 0.5 mL of thioanisole +0.25 mL of ethanedithiol per plate, incubated for three hours in a sealed container, washed with ether, air-dried in a chemical fume hood, and stored at -20°C in plastic wraps. For peptides not containing Met, Trp, or Cys, TFA containing TIPS and H_2O was used (95%, 2.5%, and 2.5%, respectively).

Peptide synthesis capacity of microtiter plate wells

TPX microtiter plates were oxidized at either room temperature or 60°C and reacted with polylysine. Peptide quantities were estimated by two different methods. In the first, the polylysine amino groups were modified with the cleavable linker HMPB [4-(4-Hydroxymethyl-3-methoxyphenoxy)-butyric acid] and FMOC-L-Cys(Acm) was coupled to the HMPB-substituted support as described above. Terminal amino groups were deprotected with 20% piperidine in NMP, washed with NMP and derivatized with dabsyl chloride. The dabsyl-amino acid derivative was released with 95% TFA, harvested from multiple wells by serial transfer, and measured by spectrophotometric scanning in a Beckman DU65 using an extinction coefficient of Dabsyl Chloride = 6.5×10^4 in TFA, λ_{max} = 495). In the second method, FMOC-L-Ala was coupled directly to the polylysine supports. The FMOC protecting group was released in 20% piperidine, harvested from multiple wells by serial transfer, and quantitated by fluor-

imetric scanning in a Perkin Elmer LS50B using E(301) = 7800 (1 mM/ml). Values for spectral constants and coefficients were determined from standards dissolved in the cleavage solvent. The solid phase capacity was 380 pMoles/well and the releasable capacity was 150 pMoles/well.

Mass Spectrometry of synthetic array peptides

A polylysine coated TPX microtiter plate was modified by preliminary reaction with FMOC-Rink Amide linker (Novabiochem) to allow peptide cleavage after synthesis using standard DCC/HOBT coupling conditions, as described above. All peptides were initiated with C-terminal Cys(Acm) to provide a constant initiation and cleavage environment. Peptides were released and deprotected by incubation for 3 hours at room temperature in 100 μL of TFA containing water, ethanedithiol and phenol, or TFA containing water and triisopropylsilane. An additional 10 uL of water and 16 hours of incubation time are required for deprotection of [bis(dimethylamino)phosphono))-tyrosine] [25]. Solutions of released peptides were concentrated by vacuum centrifugation and 0.25 μL of sample was co-crystallized with 0.25 μL of α-cyano-4-hydroxycinnamic acid in 50% ACN, 1% trifluoroacetic acid and spotted directly on a stainless steel matrix-assisted laser desorption ionization (MALDI) plate. Mass spectra were acquired using an Applied Biosystems 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA). MALDI mass spectra were externally calibrated (<20 ppm) using a standard peptide mixture.

Antibody recognition of microtiter plate arrayed peptides

Goat anti-mouse IgG (phosphatase conjugated) was purchased from Kirkegaard & Perry (Gaithersburg, MD) and Upstate Biotechnology (Lake Placid, NY). Murine monoclonal antibody 9H2, purchased from Upstate Biotechnology was prepared against a synthetic peptide containing the EGFR autophosphorylation sequence at Y1173 and was certified by the manufacturer to be specific for EGF-stimulated A431 cells by Western blot. Murine antisera prepared against phosphotyrosine were 4G10 (Upstate), ab8076 (Abcam, Ltd.), and PT01L (Oncogene Research Products). Antiserum RDI-egfract-1 was prepared against isolated tyrosine phosphorylated (activated) EGF receptor from EGF-challenged murine L-cells (Research Diagnostics, Inc.). All sera were used according to the manufacturer's recommendations and assayed by indirect ELISA. The extent of reaction was determined using a phosphatase assay kit from Kirkegaard & Perry.

Kinase assay

Peptide array substrate evaluations were performed using $p60^{c\text{-}src}$ protein-tyrosine kinase, Cat# PK03 (Oncogene Research Products) according to the instructions in the manufacturer's insert. In sequence, all wells received 30 μL of Kinase Assay Buffer (0.05 M HEPES, pH 7.5 + 0.1 mM EDTA + 0.015% BRIJ 35), 30 μL of appropriately diluted $p60^{c\text{-}src}$ in Kinase Dilution Buffer (0.1 mg/mL BSA + 0.2% β-mercaptoethanol), 30 μL of ATP mix (0.03 M $MgCl_2$ +0.15 mM ATP) in Kinase Assay Buffer. Plates were incubated at 30°C for 30 min, rinsed with distilled water and assayed for the presence of phosphotyrosine as described above. Each unit of $p60^{c\text{-}src}$ enzyme catalyzes the incorporation of one pMole of phosphate into tyrosyl residues.

List of abbreviations used

(9H2): antibody prepared against a synthetic peptide containing the EGFR autophosphorylation site at Y1173

carbonyldiimidazole (CDI)

dicyclohexylcarbodiimide (DCC)

diisopropylethylamine (DIPEA)

EGF receptor (EGFR)

ethanedithiol (EDT),

N-Hydroxybenzotriazole (HOBT)

N-methylpyrollidone (NMP)

phosphotyrosine kinase (PTK)

Polymethylpentene (TPX)

trifluoroacetic acid (TFA)

triisopropylsilane (TIPS),

Amino acid and polypeptide abbreviations were in accordance with IUPAC-IUB recommendations.

Authors' contributions

WCS designed the strategy for peptide synthesis, participated in the design of the strategy for peptide synthesis validation, carried out the peptide synthesis, testing, functional analyses, and drafted the manuscript. TPC participated in the design of the strategy for peptide synthesis validation, carried out the mass spectrometry analyses and interpreted the ms results. DJG participated in the design of the strategy for peptide synthesis validation. TDV participated in the design of the strategy for peptide synthesis validation and coordinated the mass spectrometry analyses.

Additional material

Additional File 1

EGRF (pY) peptide scanning array Vs 9H2 Antibody ELISA. EGF receptor phosphotyrosine and tyrosine overlapping peptide array sequences and ELISA test results for each peptide array.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2172-6-1-S1.pdf]

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